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Overexpression of human transforming growth factor-β1 using a recombinant CHO cell expression system

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Abstract

Transforming growth factor-β1 (TGF-β1) is secreted by most cells as a high molecular weight latent complex, which consists of latent TGF-β1 disulfide bonded to latent TGF-β1-binding protein (LTBP). Current recombinant expression systems yield less than 1–2 mg of the mature TGF-β1 per liter of cell culture medium. In an effort to produce large quantities of the recombinant cytokine for structural studies, we have constructed a mammalian expression system based on a modified pcDNA3.1(+) vector with a glutamine synthetase gene inserted for gene amplification. The leader peptide of TGF-β1 was replaced with that of rat serum albumin, and an eight-histidine tag was inserted immediately after the leader sequence to facilitate protein purification. In addition, Cys 33 of TGF-β1, which forms a disulfide bond with LTBP, was replaced by a serine residue. The resulting expression construct produced a stable clone expressing 30 mg of mature TGF-β1 per liter of spent medium. Purified TGF-β1 bound with high affinity to its type II receptor with a solution dissociation constant of ~70 nM, and was fully active in both a Mv1Lu cell growth inhibition assay and in a PAI-1 luciferase reporter assay. Owing to similarities in the synthesis, secretion, and structure of TGF-β family members, this recombinant expression system may also be applied to the overexpression of other TGF-β isomers and even to members of the TGF-β superfamily to facilitate their preparation.

Keywords: TGF-β1; LAP; LTBP; Chinese hamster ovary cell expression; Protein purification

Although TGF-β was initially described as a factor causing rat kidney fibroblasts to proliferate [1], it is now known to be a group of multifunctional cytokines with both stimulatory and inhibitory effects on a wide range of cells [2]. Currently, five distinct isoforms of TGF-β have been identified, which share 64–82% identity; three of these (TGF-β1–3) are expressed in mammalian tissues [3]. TGF-β growth factors are involved in normal tissue development such as wound healing, angiogenesis, hematopoiesis, mammary gland development, bone metabolism, and skin formation and have been implicated in multiple pathologies such as inflammatory and fibrotic diseases and tumor development [4]. Signaling via TGF-β proceeds through binding to the TGF-β type I and type II receptor serine/threonine kinases on the cell surface. This binding allows the type II receptor to phosphorylate the kinase domain of the type I receptor, which then propagates the signal through phosphorylation of Smad proteins [5].

TGF-β1 is synthesized in cells as a 390-amino acid precursor composed of a typical leader peptide and a pro-TGF-β1. The protein undergoes a number of intracellular processing steps prior to its secretion by cells. The most important processing step appears to be the proteolytic digestion of the precursor by the endopeptidase furin, which cleaves the protein at residue 278, yielding two products that assemble into dimers [6,7]. The 65–75 kDa N-terminal cleavage product corresponds to the latency-associated peptide (LAP), and the 25-kDa C-terminal portion of the precursor constitutes the mature TGF-β1 (Fig. 1). Despite cleavage of the precursor, the mature TGF-β1 remains noncovalently associated with the LAP after secretion and is released only upon activation [8]. Mature TGF-β1 contains a total of nine disulfide bonds, one of which links the monomeric

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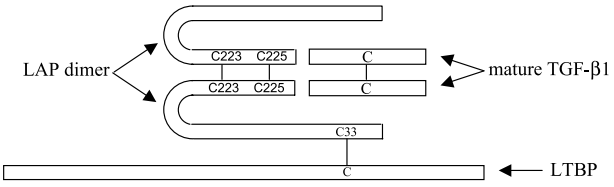

Fig. 1. Schematic representation of the latent form of TGF-β1 that is secreted by most cells. Pro-TGF-β1, which consists of noncovalently associated dimeric LAP and mature TGF-β1, is linked to LTBP by a disulfide bond through Cys 33.

subunits [9]. LAP is required not only for efficient secretion of the cytokine, but for keeping it in an inactive form, preventing it from binding to ubiquitous cell surface receptors, and maintaining its availability in a large extracellular reservoir that is readily accessed by activation. LAP contains N-linked carbohydrate while mature TGF-β1 is not glycosylated. There are three cysteine residues in each LAP subunit. Two of these, Cys 223 and 225, form interchain disulfide bonds between LAP monomers [10]. Most cells release latent TGF-β1 as a larger complex, in which TGF-β1 is associated with a 120–240 kDa glycoprotein, termed latent TGF-β1-binding protein (LTBP). LTBP is linked to LAP by a single disulfide bond with Cys 33 of TGF-β1 [11]. When recombinant human TGF-β1 is expressed in Chinese hamster ovary (CHO) cells or COS cells, however, there is no evidence that LTBP is present. In the absence of LTBP, Cys 33 is capable of forming an intramolecular disulfide bond with a cysteine residue in mature TGF-β1 [12], which prevents the release of the mature TGF-β1 from its LAP after activation. This suggests that more biologically active TGF-β1 dimer might be released from pro-TGF-β1 if Cys 33 is substituted [10].

Due to its involvement in numerous biological systems, many attempts have been made to produce recombinant TGF-β1 and its receptors using both bacterial and mammalian expression systems [12,13]. Unlike the TGF-β1 type II receptor, which also contains multiple disulfide bonds but can be expressed and refolded successfully using a bacterial system, TGF-β1 can only be expressed using mammalian cells [12,14–16]. Moreover, existing TGF-β1 expression systems have low expression levels, and purification methods involve multiple steps, further reducing the yield of the recombinant cytokine. In this work, we report a recombinant CHO-based TGF-β1 expression system capable of producing yields that are 10–30 times higher than those previously reported.

Materials and methods

Enzymes, media, and chemicals

*Xma*I and *Bsp* 119I were from Fermentas (Hanover, MD). *Bam*HI, *Xho*I, and T4 DNA ligase were from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). DMEM/F12 medium, fetal bovine serum (FBS), CHO-S-SFM II serum-free medium, OPTI-MEM I medium, and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Glutamine-free GMEM-S medium and GS supplement were from JRH Biosciences (Lenexa, KS). Ni–NTA agarose was from Qiagen (Valencia, CA). Chemicals were from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

Cell line and culture conditions

The Chinese hamster ovary cell line CHO-lec3.2.8.1 was kindly provided by Dr. Pamela Stanley, Albert Einstein College of Medicine, NY. Under nonselective conditions, cells were grown in DMEM/F12 medium containing 5% FBS. Selection was performed in glutamine-free GMEM-S medium supplemented with GS supplement, 5% dialyzed FBS, and a specified amount of methionine sulfoximine (MSX). For TGF-β1 protein expression, CHO-S-SFM II serum-free medium was used.

Plasmid construction

To construct a glutamine synthetase (GS)-containing vector, the coding region of Chinese hamster GS was amplified by PCR using a CHO-K1 cDNA library (Stratagene) as a template. The forward and reverse primers were 5′-GCG ATA CCC GGG TAT ACC ATG GCC ACC TCA GCA AGT-3′ and 5′-CGG GTG TTC GAA TTA GTT TTT GTA TTG GAA GGG-3′ (the underlined nucleotides of the primers denote the *Xma*I and *Bsp* 119I sites, respectively). The PCR product was digested with *Xma*I and *Bsp* 119I restriction enzymes and then ligated to pcDNA3.1(+) (Invitrogen), which was previously digested with these two enzymes. This replaced the neomycin gene in pcDNA3.1(+) with the GS open reading frame, permitting amplification of the vector using MSX. This vector was named pcDNA-GS. Next, TGF-β1 cDNA was inserted into this vector to create pcDNA-GS-TGF-β1. In brief, mutant porcine TGF-β1 cDNA (kindly provided by Dr. Seong-Jin Kim, National Institutes of Health, Bethesda, MD), in which Cys 223 and Cys 225 were mutated to serines, was used as a template to amplify TGF-β1. The amino acid sequence of mature porcine TGF-β1 is identical to that of human mature TGF-β1. Two successive runs of PCR were performed. The four oligonucleotides used were: TGF1, 5′-GGT TCT GCC TTT TCT CAC CAC CAT CAC CAC CAC CAT CAT CTG TCC ACC TGC AAG AC-3′; TGF2, 5′-TAG T CTC GAG TTA TCA GCT GCA CTT GCA GG-3′; RSA1, 5′-AAA GGG GGA TCC GCC ACC ATG AAG TGG GTA ACC TTT CTC CTC CTC-3′; and RSA2, 5′-AGA AAA

GGC AGA ACC GGA GAT GAA GAG GAG GAG GAG AAA GGT TAC-3′. In the first amplification, primers TGF1 and TGF2 were used in concert with porcine TGF-β1 cDNA as the template. The second amplification was performed using the first PCR product as a template and RSA1, RSA2, and TGF2 as primers. This second amplification yielded a product encoding the rat serum albumin leader sequence MKWVTFLLLLFISGSAFS followed by eight histidine residues and pro-TGF-β1 at the 3′ end. It also introduced a *Bam*HI site at the 5′ end and an *Xho*I site at the 3′ end. This segment was digested with *Bam*HI and *Xho*I and inserted between the *Bam*HI and *Xho*I sites of pcDNA-GS. To mutate the serine residues at positions 223 and 225 of TGF-β1 back to cysteines, the oligonucleotides 5′-CGC CTC AGT GCC CAC TGT TCC TGT GAC AGC AAA GAT AAC-3′ and 5′-GTT ATC TTT GCT GTC ACA GGA ACA GTG GGC ACT GAG GCG-3′ were used in conjunction with the QuickChange Site-Directed Mutagenesis kit (Stratagene). A further mutation was introduced to replace Cys 33 of TGF-β1 with serine using the primers 5′-GGA TCC CTG TCC ACC TCC AAG ACC ATC GAC ATG-3′ and 5′-CAT GTC GAT GGT CTT GGA GGT GGA CAG GGA TCC-3′. The resulting expression vector was confirmed by DNA sequencing and was termed pcDNA-GS-TGF-β1.

Establishment of a stable TGF-β1-expressing cell line

CHO-lec3.2.8.1 cells were trypsinized and seeded in a T-25 flask so that they were near confluence on the day of transfection. Immediately before transfection, the medium was replaced with 4 ml fresh DMEM/F12 medium containing 5% FBS. The vector pcDNA-GS-TGF-β1 (10 μg) and 30 μl of Lipofectamine 2000 were each diluted into 0.5 ml OPTI-MEM I medium, and then combined and allowed to incubate at room temperature for 20 min. This mixture was then added to the culture. The medium was replaced with fresh medium after 24 h. After two days had elapsed since transfection the cells were trypsinized, seeded into ten 10 cm culture dishes, and cultured with glutamine-free GMEM-S medium supplemented with GS supplement, 5% dialyzed FBS, and 30 μM MSX. After about three weeks, colonies were transferred into 48-well plates containing 0.5 ml selection medium. When the cultures reached confluence, supernatants were taken and assayed for TGF-β1 expression by ELISA. The 10 highest expressing clones were subjected to the next round of selection by further splitting into 10 cm dishes containing 150 μM MSX selection medium. The third round of selection was made in the presence of 500 μM MSX. After three rounds of selection and amplification, the clone with the highest expression of TGF-β1 was chosen for large-scale recombinant protein production.

TGF-β1 expression and purification

To generate TGF-β1-containing medium, the chosen clone was transferred to T-500 triple layer flasks after expansion and cultured in glutamine-free GMEM-S medium, supplemented with GS supplement, 5% dialyzed FBS, and 500 μM MSX. When the cultures reached confluence, cells were washed twice with Hanks' solution and cultured with CHO-S-SFM II serum-free medium. After 3–4 days, the medium was harvested and replaced with fresh serum-free medium. The harvest medium was filtered with a 0.22 μm cellulose acetate filter and stored at −20 °C for future use. A column containing 20 ml Ni–NTA agarose was equilibrated with loading buffer containing 50 mM Tris–HCl and 150 mM NaCl at pH 8.0. Approximately 500 ml harvest medium was thawed, refiltered, and applied to the Ni–NTA column. The column was then washed with 30 ml of loading buffer and the protein was eluted by a linear concentration gradient of imidazole. Eluted protein fractions were pooled and the pH was adjusted to 3.0. The sample was concentrated twofold, filtered, and applied to a HiLoad 16/60 Superdex 200 preparative grade column (Amersham Biosciences, Piscataway, NJ) with 50 mM glycine, 50 mM NaCl, pH 4.0, as the running buffer.

TGF-β1 content and protein concentration assays

TGF-β1 content was measured by ELISA using a DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN). Briefly, 0.2 μg of mouse anti-human TGF-β1 antibody was immobilized onto each well of an ELISA plate. Chicken anti-human TGF-β1 antibody was used as a detection antibody at a concentration of 300 ng/ml. Samples were acid-activated prior to the assay, which was performed according to the manufacturer's instructions. Protein concentration was determined by using a bichinchoninic acid (BCA) protein assay kit (Sigma–Aldrich).

SDS–PAGE and Western blot assays

SDS–PAGE was performed using either homogeneous 20% polyacrylamide gels with a PhastSystem apparatus (Amersham Biosciences) or 12.5% SDS–polyacrylamide ReadyGel precast gels (Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue or silver stain according to the manufacturer's specifications. Western blot assays were performed as described by Sambrook et al. [17]. Mouse anti-human TGF-β1 antibody (R&D Systems) was used as the primary antibody at a concentration of 2 μg/ml in conjunction with a goat anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) at a concentration of 1:20,000. The peroxidase substrate was 3-amino-9-ethylcarbazole (Sigma–Aldrich).

N-terminal sequencing and mass spectral analysis

N-terminal amino acid sequencing was performed by repeated Edman degradation using a Model 477A protein sequencer coupled to a Model 120A PTH analyzer (Perkin–Elmer/Applied Biosystems) at the NIAID core facility. Electrospray ionization (ESI) mass spectroscopy (MS) measurements were acquired and recorded with a Perkin–Elmer Sciex API-300 LC/MS/MS system (Perkin–Elmer/Applied Biosystems, Foster City, CA). Protein samples exchanged into a volatile buffer and at a concentration of 10 pM/μl or greater were delivered by direct infusion using an infusion pump in a carrier solution of 30% acetonitrile, 1% acetic acid at a rate of 5 μl/min through a fused-silica capillary of 100-μm internal diameter. When necessary, samples were desalted through an in-line desalting trap cartridge.

Binding measurements

TGF-β1 binding to the TGF-β1 type II receptor was first assayed using the Quantikine Human TGF-β1 Immunoassay kit (R&D Systems) in which recombinant human TGF-β1 type II receptor was coated on the plate. TGF-β1 standard from the kit was used for comparison. Optical density was measured at a wavelength of 450 nm. Test was performed according to the manufacturer's protocol.

Surface plasmon resonance (SPR) measurements were performed using a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden). Preparation of the TGF-β1 type II receptor (residues 22–136) was similar to that described previously [15]. In brief, 25 mg of inclusion bodies was dissolved in 6 M guanidine hydrochloride and injected into 1 L of a refolding solution that contained 0.5 M arginine, 20 mg/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) protease inhibitor, 2 mM EDTA, 5 mM cysteamine (reduced form), 0.5 mM cystamine (oxidized form), and 0.1 M Tris–HCl, pH 8.0. The solution was stirred for 24 h at 10 °C and then dialyzed against 5 mM Tris–HCl, pH 8.5. After filtering, the refolding solution was loaded onto a Source 15Q column (Amersham Biosiences), which was pre-equilibrated with 5 mM Tris–HCl, pH 8.5. Two major protein peaks were eluted by a linear gradient from 0 to 1 M NaCl. Fractions corresponding to the first peak were pooled and concentrated and then purified with a Superdex 200 column.

TGF-β1 was immobilized on two flow cells of a CM5 sensor chip at concentrations of 0.7 and 1.4 μM, respectively, in 50 mM sodium acetate, pH 5.0, using *N*-hydrosuccinimide/1-ethyl-3(-3-dimethylaminopropyl)-carbodiimide hydrochloride (NHS/EDC) at a flow rate of 20 μl/min. The running buffer and the analyte sample buffer were 10 mM Tris–HCl, pH 8.0. Binding of the TGF-β1 type II receptor to the immobilized TGF-β1 was measured using serial dilutions of the receptor from 150 to 0.586 nM at a flow rate of 20 μl/min. The chip surface was regenerated with 10 mM glycine, pH 3.0, at a flow rate of 30 μl/min for 30 s, followed by buffer stabilization for 3 min. The dissociation constant (K_D) was derived from a linear regression of steady state of 1/Response versus 1/C double reciprocal plots as well as by fitting of binding kinetics using a first-order Langmuir model.

TGF-β1 bioassays

Two methods were used to assess the biological activity of recombinant TGF-β1. In the first assay, mink lung cells (Mv1Lus) were used to perform a growth inhibition assay [18]. As a second means of assessing biological activity, mink lung epithelial cells (MLECs) transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct were utilized [19]. Briefly, the transfected MLECs were plated in 96-well tissue culture dishes (1.6×10^4 cells/well) and were allowed to attach for 3 h at 37 °C in a 5% CO_2 incubator. DMEM–0.1% BSA containing TGF-β1 was added. After 14 h at 37 °C, cell extracts were prepared and assayed for luciferase activity using the enhanced luciferase assay kit (Analytical Luminescence, San Diego, CA). Luciferase activity was reported as relative light units. Commercial TGF-β1 (R&D Systems) was used for comparison.

Results

Construction of a TGF-β1 expression plasmid

The expression process of TGF-β1 is distinct from those of other proteins, as it is synthesized and secreted as a homodimeric latent form. Mature TGF-β1 can be released from the noncovalently associated LAP through an activation process by either extreme pH or other methods [20]. In addition to TGF-β1, most cells also secrete LTBP disulfide bonded with LAP through Cys 33. The absence of LTBP in the recombinant CHO TGF-β1 expression system, however, may complicate the release of mature TGF-β1 due to potential disulfide mis-pairing between Cys33 and cysteines of mature TGF-β1 [14]. To promote release of mature TGF-β1, we introduced a Cys-to-Ser mutation at position 33 in the porcine TGF-β1 cDNA, which is 94% identical in the precursor and 100% identical in the mature part to human TGF-β1. To streamline purification, we also inserted an eight-histidine tag immediately after the leader sequence. Since the tag was fused to the latency peptide, it did not affect the amino acid sequence of the mature TGF-β1 expressed, but enabled use of Ni–NTA-based metal affinity chromatography in purification.

It is known that leader sequences play an important role in protein secretion. Some heterologous leader sequences can greatly increase protein expression levels in eukaryotic or prokaryotic cells [21,22]. Since physiological levels of TGF-β1 are low, it is possible that the endogenous leader sequence is not optimal for high-level recombinant protein expression. As serum albumin represents one of the most abundant proteins secreted by animals, in an attempt to further optimize the TGF-β1 expression, we replaced the generic TGF-β1 leader sequence with that of rat serum albumin.

Expression and purification of TGF-β1

After three rounds of selection using MSX, one clone (clone 50) was cultured in T-flask using selection medium. When cells were confluent, the medium was replaced with serum-free medium and cultured for 3–4 days to allow accumulation of TGF-β1. The concentration of TGF-β1 in the harvest medium was about 30 mg/L as determined by ELISA. Due to the high and specific affinity of His-tagged proteins for Ni–NTA, the harvest medium could be directly loaded onto the Ni–NTA agarose column without an initial concentration step. The eluted protein from the column mainly consisted of pro-TGF-β1, giving rise to three major bands on an SDS–PAGE gel with approximate apparent molecular weights of 100, 75, and 25 kDa, which represented pro-TGF-β1, LAP, and mature TGF-β1, respectively (Fig. 2). N-terminal sequencing of pro-TGF-β1 and LAP resulted in a peptide of HHHHHHHHLSTSKTIDMELV, indicating that the leader sequence was cleaved just before the histidine tag. The presence of eight-histidine residues in the expressed protein did not appear to interfere with synthesis or secretion. Ni–NTA affinity-eluted TGF-β1 was subjected to low pH treatment in order to release the mature TGF-β1 from its LAP. Mature TGF-β1 was then purified to homogeneity using a size exclusion column under low pH conditions (Figs. 3 and 4A and B). The elution profile showed that TGF-β1 was eluted at a

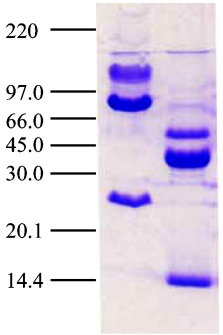

Fig. 2. SDS–PAGE analysis of TGF-β1 purified by Ni–NTA chromatography. Samples were loaded onto a homogeneous 20% SDS–polyacrylamide gel and stained with Coomassie blue. Lane 1, nonreducing conditions and lane 2, reducing conditions. The three major bands visible correspond to pro-TGF-β1, LAP, and mature TGF-β1.

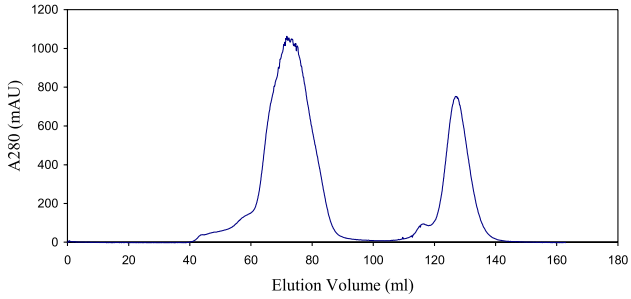

Fig. 3. Purification of recombinant TGF-β1 by size exclusion chromatography. Following purification by Ni–NTA agarose, TGF-β1 was acid-activated and concentrated twofold. The sample (2 ml) was loaded onto a HiLoad 16/60 Superdex 200 preparative grade column with a running buffer of 50 mM glycine, 50 mM NaCl, pH 4.0. Pro-TGF-β1 and LAP were eluted in the first major peak. The small shoulder preceding the second major peak was determined to be non-covalently linked mature TGF-β1 dimer (data not shown). The second major peak represents mature TGF-β1.

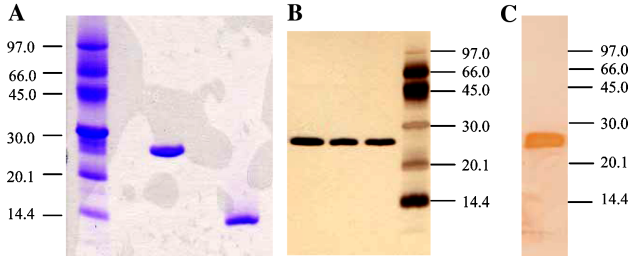

Fig. 4. Electrophoretic and Western blot analysis of purified TGF-β1. (A) SDS–PAGE analysis of recombinant, mature TGF-β1 purified by Ni–NTA chromatography and size exclusion chromatography. Lane 1, molecular weight markers (kDa); lane 2, nonreducing conditions; and lane 3, reducing conditions. (B) Purity analysis of recombinant, mature TGF-β1 by silver staining. Lanes 1–3 contain 2, 1.5, and 1 μg, respectively, of purified TGF-β1 electrophoresed under nonreducing conditions on a 20% SDS–polyacrylamide gel; and lane 4, molecular weight markers. (C) Western blot analysis. Purified TGF-β1 (1.5 μg) was electrophoresed under nonreducing conditions on a 12.5% SDS–polyacrylamide gel, transferred by electroblotting to a PVDF membrane, and visualized by immunostaining as described in the text.

smaller apparent molecular weight. This phenomenon, also observed by other groups [14], could be due to the hydrophobic interaction between TGF-β1 and the chromatographic matrix.

After only two steps of purification, 10 mg of pure TGF-β1 was obtained from 500 ml harvest medium (Table 1). The identity of the purified mature TGF-β1 was further confirmed by Western blot assay (Fig. 4C) and by N-terminal sequencing (ALDTNYCFSSTEKNCCVRQL). Mass spectrometry measurements gave a molecular weight of 25574.0 Da, which is in close agreement with the predicted value of 25571.6 Da.

Binding of recombinant TGF-β1 to its type II receptor

Direct binding of TGF-β1 to recombinant human TGF-β1 type II receptor was measured using both

Table 1
Purification of recombinant TGF-β1

Step	Volume (ml)	Protein (mg)[a]	TGF-β1 (mg)	Purification (fold)	Recovery (%)
Harvest medium	500	605	15.4[b]	1.0	100
Ni–NTA	28	42.4	11.2[b]	10.4	72.7
Superdex 200	126	10.6	10.6[a]	40.0	68.8

[a] Protein estimated by BCA protein assay.
[b] TGF-β1 estimated by ELISA.

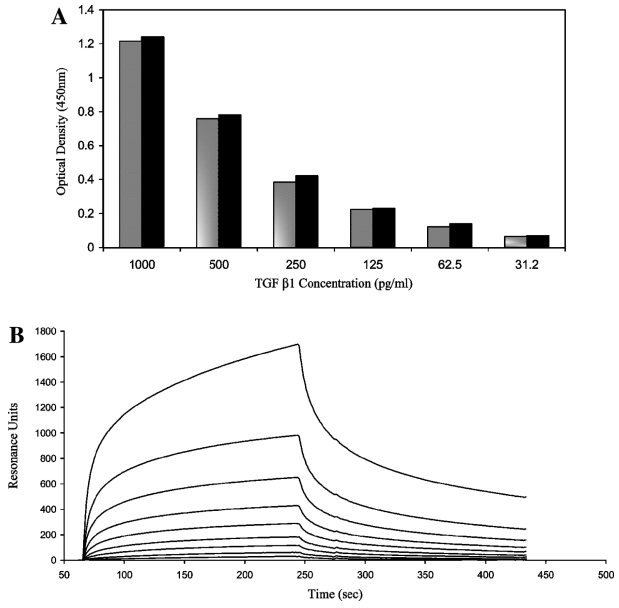

Fig. 5. Binding of TGF-β1 to the TGF-β1 type II receptor. (A) Serial dilutions of purified TGF-β1 (black bars) and commercial TGF-β1 (hatched bars) were added to plates coated with human recombinant TGF-β1 type II receptor and analyzed by ELISA. (B) BIAcore sensorgram of the TGF-β1 type II receptor binding to TGF-β1 immobilized on a CM5 chip. The concentrations of the type II receptor used were 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17, and 0.586 nM.

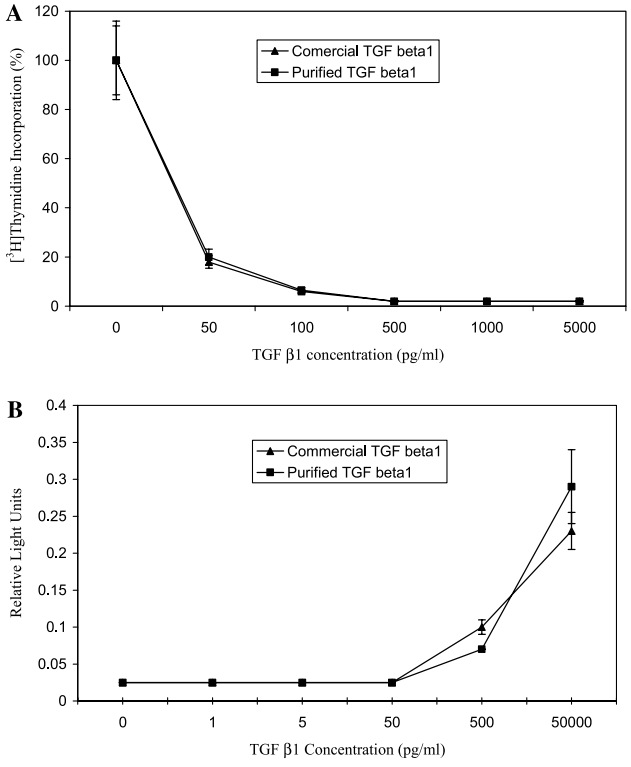

Fig. 6. Biological activity of purified TGF-β1. All assays were performed in triplicate, with error bars representing the standard error of the mean of the samples. Comparisons of purified and commercially available TGF-β1 were performed by growth inhibition assays in Mv1Lu cells (A) and by enhancement of luciferase expression in recombinant MLECs (B).

ELISA and BIAcore techniques. Assessments by ELISA showed that the binding affinity of the purified TGF-β1 to the type II receptor was equal to that of commercially prepared TGF-β1 (Fig. 5A). Surface plasmon resonance measurements by BIAcore indicated that the apparent dissociation constant (K_D) of TGF-β1 with the type II receptor was 70 nM, which is similar to the value of 100 nM reported previously [23]; the on and off kinetic constants were 8.1×10^4 1/ms and 5.9×10^{-3} 1/s, respectively (Fig. 5B).

Biological activity of recombinant TGF-β1

To determine whether the purified TGF-β1 is biologically active and to compare its activity with commercial TGF-β1, bioassays were conducted to measure the ability of the recombinant protein to inhibit the growth of Mv1Lu cells (Fig. 6A) and to induce luciferase expression in recombinant mink lung epithelial cells (Fig. 6B). In both assays, the dose–response curves showed that the biological activity of the purified TGF-β1 was comparable to that of the commercially prepared TGF-β1.

Discussion

TGF-β was first purified from human platelets, with extremely low yields [24]. Subsequently, several groups have successfully expressed recombinant TGF-β1 in CHO cells using wild-type TGF-β1 cDNA [12,15]. The expression levels of these recombinant constructs varied from less than one to several milligram/liter, even with use of roller bottles or bioreactors. In addition, due to the lack of a cost-effective affinity purification method, previous recombinant systems necessitated lengthy, multi-step purification protocols that further reduced yields to less than 1–2 mg/L.

We have designed a CHO-based expression vector to overexpress high amounts of recombinant human TGF-β1. The vector was modified from the parental pcDNA3.1(+) vector by insertion of a glutamine synthetase gene to facilitate the amplification of the recombinant plasmid in CHO cells. To improve the production of TGF-β1, a Cys 33–Ser mutation was created in the TGF-β1 latency region and the natural leader sequence was replaced with that of rat serum albumin. Finally, an eight-histidine tag was inserted immediately after the signal peptide to greatly simplify purification without affecting the amino acid composition of the mature TGF-β1. Use of our pcDNA-GS-TGF-β1 vector resulted in expression levels of 30 mg/L of spent medium and permits use of a simple two-step purification scheme with a final yield of over 20 mg of purified protein per liter. This represents a 10–30-fold improvement in recombinant TGF-β1 preparation as compared with previous efforts.

It is believed that LTBP plays an important role in TGF-β1 folding and secretion in some cells [25]. Our results indicate, however, that the absence of LTBP does not appear to adversely affect TGF-β1 folding and secretion in CHO cells. Additionally, although we presume that the rat serum albumin leader sequence may have an effect on the high expression level, its specific contribution was not determined as no control construct was made.

Members of the TGF-β family share a conserved gene structure that consists of both latent and mature regions. The sequence identity among the five isoforms (TGF-β1–5) is generally greater than 50%. In particular, the cysteine residues in both the latent and mature regions of the sequences (including Cys 33) are conserved throughout the isoforms in all species. All TGF-β isoforms are synthesized as precursors that are cleaved intracellularly. After secretion, the latent growth factor, which contains the mature portion noncovalently linked to LAP, also remains bound to LTBP [25]. It is therefore possible that the TGF-β1 expression strategies developed here may be applied to other TGF-βs of various species. It is not clear whether the current expression system may also be used to achieve overexpression of growth factors other than TGF-β that are nonetheless members of the TGF-β superfamily, such as bone morphogenetic proteins (BMP). Although BMP is also expressed in precursor form consisting of an N-terminal pro-peptide and a C-terminal mature growth factor, the overall sequence identity between BMP and TGF-β is about 30%. Furthermore, the conserved cysteine residues in the TGF-β LAP are not conserved in BMP, suggesting that the latency peptide of BMP may adopt a different structure than that of TGF-β. Nevertheless, the structural similarity between mature BMP and TGF-β1 may permit the expression of mature BMP as a chimeric molecule using the TGF-β1 LAP.

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